Versican/PG-M Isoforms in Vascular Smooth Muscle Cells

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Abstract—The expression of increased amounts of proteoglycans in the extracellular matrix may play a role in vascular stenosis and lipid retention. The large chondroitin sulfate proteoglycan versican is synthesized by vascular smooth muscle cells (SMCs), accumulates during human atherosclerosis and restenosis, and has been shown to bind LDLs. We recently demonstrated that adult rat aortic SMCs express several versican mRNAs. Four versican splice variants, V0, V1, V2, and V3, have recently been described, which differ dramatically in length. These variants differ in the extent of modification by glycosaminoglycan chains, and V3 may lack glycosaminoglycan chains. In this study, we characterized versican RNAs from rat SMCs by cloning, sequencing, and hybridization with domain-specific probes. DNA sequence was obtained for the V3 isoform, and for a truncated V0 isoform. By hybridization of polyadenylated RNA with domain-specific probes, we determined that the V0, V1, and V3 isoforms are present in vascular SMCs. We confirmed the presence of the V3 isoform in polyadenylated RNA and in RT-PCR products by hybridization with an oligonucleotide that spans the splice junction between the hyaluronan-binding domain and the epidermal growth factor-like domain. In addition, a novel splice variant was cloned by PCR amplification from both rat and human SMC RNA. This appears to be an incompletely spliced variant, retaining the final intron. PCR analysis shows that this intron can be retained in both V1 and V3 isoforms. The predicted translation product of this variant would have a different carboxy-terminus than previously described versican isoforms. (***Arterioscler Thromb Vasc Biol.* 1999;19:1630-1639.**)

Key Words: proteoglycan-M ■ proteoglycan ■ splicing ■ vascular smooth muscle ■ unspliced

The major proteoglycan present in the interstitial matrix of vascular smooth muscle cells (SMCs) is a large chondroitin sulfate (CS) proteoglycan that forms aggregates with the glycosaminoglycan hyaluronan.[1] This proteoglycan has been identified by immunohistochemistry, immunoblotting, and cloning experiments as versican.[2] Versican, also known as PG-M, is a member of a gene family that also includes aggrecan, the predominant proteoglycan of cartilage, and 2 brain proteoglycans, neurocan and brevican.[3] These proteoglycans bind hyaluronan via the amino-terminal globular domain (G1) comprising an immunoglobulin-like domain and a proteoglycan tandem repeat. The selectin-like domain (G3) at the carboxy-terminus is composed of epidermal growth factor (EGF)-like, lectin-like, and complement-regulatory protein (CRP)-like domains.[3] The G3 domain of versican has been shown to bind tenascin-R, as well as several carbohydrates.[4–6] Between the globular domains is a larger extended region bearing CS chains. These CS chains are believed to serve an antiadhesive function, and their hygroscopic properties make versican a large space-filling molecule that may resist the compression of pulsing blood.[1,7,8]

The central glycosaminoglycan (GAG)-attachment domains of versican are encoded by exons that can undergo differential splicing.[9,10] The core proteins translated from the splice variants are predicted to differ greatly in length and in the number of CS chains attached.[11–13] These variants are known as V0, which contains both αGAG and βGAG exons, V1, containing the βGAG exon, V2, having the αGAG exon, and V3, consisting only of the globular domains.

We have previously shown that the V1 isoform is expressed by monkey and human vascular SMCs in vitro and in vivo.[2] We recently showed that several RNA bands from total rat adult SMC RNA hybridize at high stringency to versican cDNA probes[14] and thus appear to be products of the versican gene. These RNAs could include known and previously undescribed splice variants, or those using different initiation or termination sites. Identification of protein domains that may be differentially regulated should allow us to ask more specific questions about the function of those domains, and to develop tools (eg, antibodies, recombinant expression for specific domains) to answer those questions.

Methods

Cell Culture and RNA Isolation

Rat SMCs were cultured in modified Waymouth's medium containing 5% calf serum, as previously described,[15] and harvested on reaching confluence. The rat SMC lines WKY3M-22, WKY12-22,

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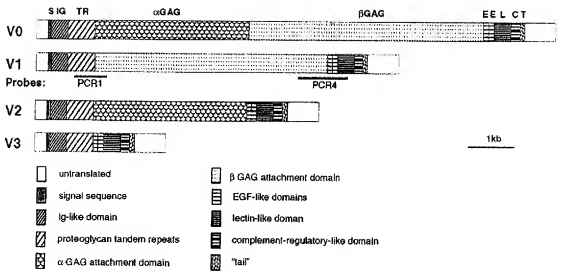

Figure 1. Domain structure of human versican isoforms[12,13,30] and the probes used for cloning rat versican. Monkey PCR1 and human PCR4 probes were previously cloned.[1] GAG indicates glycosaminoglycan.

SD3M-23, and SD12 were previously established and characterized.[14–18] Human aortic SMC line RR 87-80 (gift of Dr Russell Ross) was established from a 2- to 3-month-old child who died of sudden infant death syndrome.[19] Human SMCs were cultured in DMEM containing 10% FCS. After culture in DMEM containing 1% human plasma-derived serum for 48 hours, cells were stimulated with 10 ng/mL platelet-derived growth factor for 24 hours. Total RNA was isolated as previously described.[20] Polyadenylated [poly(A)$^+$] RNA was isolated by oligo(dT) chromatography (Stratagene).

Electrophoresis, Northern, and Southern Transfer

Fifteen micrograms of total RNA or 10 μg of poly(A)$^+$ RNA was separated on 0.8% agarose gels containing formaldehyde.[21] RNA band sizes were determined in comparison with an RNA ladder (Life Technologies) probed with radiolabeled λ*Hin*dIII DNA. RNA in the gels was subjected to limited alkaline hydrolysis, transferred to Zetaprobe (Bio-Rad) membrane, and immobilized by UV cross-linking.[21] DNA was separated in agarose gels buffered with Tris-borate–EDTA (TBE), depurinated for 10 minutes in 0.25 mol/L HCl, transferred to Zetaprobe,[21] and immobilized by UV cross-linking.

Preparing and Screening cDNA Library

RNA was isolated from confluent cultures of the rat SMC cell line WKY3 M-22, and poly(A)$^+$ RNA was isolated. A cDNA library [primed with oligo(dT)] in λZAPII (Stratagene) was prepared.

Primer Sequence and Position Relative to Rat and Human Versican cDNAS*

Primer Name	Sequence (5′–3′)	Species/ Isoform	Start	End	Domain†
09V	GGAACTTCACGCAGCTT	Rat V3	1123	1139	HABR
10V	GTCCTTTGGTATGCAGA	Rat V3	2139	2123	Tail
		Human V1	7387	7371	Tail
11V	CAGCGGAAGTCATGTTC	Rat V3	1773	1757	Lectin
16Bprime	CTTTGACCAGTGCGATTACG	Rat V3	1150	1169	HABR
		Human V1	1136	1155	HABR
17Bprime	GCAGTAGGCATCAAATCTGC	Rat V3	1315	1296	HABR
		Human V1	1301	1282	HABR
18VSPLR	GAGATCAGGTCGTTTAAAGCAG	Rat V3	1333	1312	HABR/EGF
19VSPLH	GCGATCAGGTCGTTTAAAGCAG	Human V3	1158	1137	HABR/EGF
21VE5	GGAGGCACCTGTTATCCTAC	Human V1	6606	6625	EGF
22VU3	TCTCGAGGTGATAGGAAAGG	Human V1	7574	7555	3′-UTR
24VL3	CAGGGAACATCATTCCACTG	Human V1	7147	7128	Lectin
26VBG	GTGGTTGTCACATCAGTAGC	Human V1	1519	1500	βGAG
27L	GACTATGGCTGGCACAA	Rat V3	1565	1581	Lectin
29E1415	TATGCAGATGGGTTCATGCA	Rat V3	2130	2111	CRP/tail
36BG	ATCTGAGAAACAAGTGTCCC	Rat V0	6068	6087	βGAG
VC21R	CACAACAGTATTACTTGCTCC	Rat Int14	113	94	Int14

*GenBank accession numbers: rat V3, AF072892; rat V0, AF062402; rat Intron 14, AF084544; human V1, X15998; and human V3, D32039.

†Domain abbreviations: βGAG indicates β-glycosaminoglycan attachment; CRP; complement-regulatory protein-like; EGF, epidermal growth factor–like; HABR, hyaluronan-binding region; Int14, intron 14; Lectin, lectin-like; and 3′-UTR, 3′-untranslated region.

Plaques (3×10^5) were screened for clones that hybridized to both PCR1 and PCR4 versican cDNAs,[2] which contain sequences that are present in all known versican splice variants (Figure 1); 3 clones (rVa, rVd, and rVe) that hybridized with both probes, and 1 (rVb) that hybridized with only the 3′ probe, were rescued from the λZAPII clones (protocol supplied by Stratagene), subjected to restriction mapping and sequenced.

Probes and Hybridization

Monkey versican PCR1 and human versican PCR4 cDNAs[2] were isolated from vector DNA by restriction digestion before labeling. An *EcoRI/SacI* fragment representing nucleotides 1 to 631 of rat versican plasmid rVb (see below) was used as a probe for the αGAG domain. A *PstI/EcoRI* fragment representing nucleotides 1315 to 2148 of rVb was used as a probe for the βGAG domain. Probes for the 5′- and 3′-domains of versican were prepared by PCR, using rat versican cDNAs as templates. Reaction solutions were as described by the supplier (Perkin Elmer). For the hyaluronan-binding region (HABR) probe, primers 16B′ and 17B′ were used to amplify the second proteoglycan tandem repeat (see Table, for oligonucleotide sequences). Oligonucleotides were synthesized by Operon, Life Technologies, or National Biosciences. Plasmid rVd (10 ng/mL) template was amplified for 30 cycles (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute). For the G3 domain probe, primers 21VE5 and 24VL3 (see the Table) were used to amplify most of the EGF-like and lectin-like domains. Plasmid rVa (10 ng/mL) was amplified for 30 cycles (94°C for 1 minute, 43°C for 1 minute, and 72°C for 1 minute). After PCR, primers were removed from the desired product by using a Centricon 50 filter apparatus (Millipore). Restriction fragments and PCR products were labeled by random priming in the presence of [α-^{32}P]dCTP (ICN) and hybridized to northern and Southern blots as previously described.[22]

Oligonucleotide probes 19VSPLH (human) and 18VSPLR (rat) (see the Table) were designed to be antisense to the putative splice junction between the HABR and the first EGF-like domain. Labeled oligonucleotides were prepared by using polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP,[23] and hybridized as previously described.[16] Posthybridization washes were 2×5 minutes and 1×20 minutes in 2×SSPE 3.47 mmol/L (0.1%) SDS at 42°C and 1×20 minutes 0.6×SSPE 3.47 mmol/L (0.1%) SDS at 45°C (1×SSPE=0.15 mol/L NaCl, 0.2 mol/L NaH_2PO_4, and 0.02 mol/L Na$_2$EDTA). Under these conditions, the oligonucleotides would hybridize to molecules where both halves of the splice junction are adjacent to each other; the rat V3 clones (including rVa, rVd, and rVe) hybridized to the probe, but the larger clone, rVb, corresponding to most of the V0 isoform, did not (not shown).

RT-PCR Analysis

Total RNA was reverse-transcribed by using Superscript II (Life Technologies). Rat and human SMC total RNAs (0.56 μg and 1 μg, respectively) were reverse-transcribed by using random hexamer primers. Versican sequences were amplified as previously described,[16] using either a kit from Perkin-Elmer or Taq DNA polymerase from Stratagene, nucleotides from Life Technologies, and primers as indicated in Results. Products were analyzed on TBE gels, stained with ethidium bromide, photographed, and transferred to Zetaprobe membrane as described above.

Cloning of RT-PCR Products

Primers 27L and 10V were used to amplify reverse-transcribed rat and human SMC RNA. RT-PCR products were cloned by using the TA cloning kit from Invitrogen and sequenced.

DNA Sequencing and Sequence Analysis

DNA sequence analysis was performed by several methods, ie, by an automated method with fluorescently tagged terminators (Taq Dyedeoxy Terminator Cycle Sequencing Kit, Applied Biosystems) and gel analysis on an Applied Biosystems 373A DNA sequencer, or by using [^{35}S] α-*thio*-dCTP and a Sequenase 2.0 kit from Life Technologies, or a Sequiterm Cycle Sequencing Kit (Epicentre). "Long Ranger" gel solutions (JT Baker) were used for analysis of ^{35}S-labeled reactions, and dried gels were exposed to X-Omat AR or Biomax film (Eastman Kodak). Sequence was compiled by using GCG software.[23]

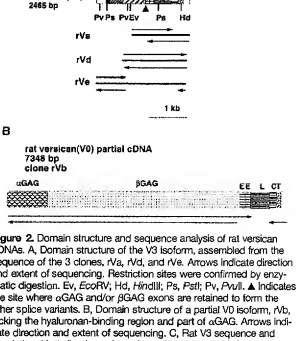

Figure 2. Domain structure and sequence analysis of rat versican cDNAs. A, Domain structure of the V3 isoform, assembled from the sequence of the 3 clones, rVa, rVd, and rVe. Arrows indicate direction and extent of sequencing. Restriction sites were confirmed by enzymatic digestion. Ev, *EcoRV*; Hd, *HindIII*; Ps, *PstI*; Pv, *PvuII*. ▲ Indicates the site where αGAG and/or βGAG exons are retained to form the other splice variants. B, Domain structure of a partial V0 isoform, rVb, lacking the hyaluronan-binding region and part of αGAG. Arrows indicate direction and extent of sequencing. C, Rat V3 sequence and translation. Underlined are sites of possible N-linked (NXS and NXT) and O-linked glycosylation (T), possible glycosaminoglycan chain attachment (SG and GS), and polyadenylation consensus sequence (AATAAA) sites. / indicates predicted signal sequence cleavage; *, 3′-termini of rVd and rVe clones.

The size of the predicted translation product was determined by using GCG software,[23] and the position of signal cleavage was predicted by using Signal P V1.1 software.[24] O-linked polysaccharide addition was predicted by using the NetOGlyc V2.0 software.[25–27]

Results

Cloning of V3, a Small Versican Isoform

To determine which versican protein coding regions are included in each of the versican RNAs expressed by adult rat smooth muscle cells, rat SMC cDNAs containing 5′- and 3′-versican domains were cloned (see Methods). Three small clones, rVa, rVd, and rVe, were subjected to restriction mapping and sequence analysis (see Figure 2A and 2C, Genbank Accession No. AF072892). The high degree of protein sequence identity between these molecules and mouse (96%) and human (91%) versican/PG-M amino- and carboxy-terminal globular domains[28] supports their identification as rat versican clones. The regions of homology included amino-terminal HABR, and the entire G3 domain, that is, the EGF-like, lectin-like, and CRP-like regions through the translation stop. The HABR is immediately apposed to the first EGF-like domain and no sequences with homology to either of the alternately spliced exons (αGAG or βGAG) that encode the attachment sites for glycosaminoglycans are present. The length of the assembled sequence is 2465 bp. Zako et al[12] have demonstrated the existence of this isoform in mice and humans and refer to it as V3.

A partial V0 isoform clone, rVb, was also sequenced (Figure 2B; Genbank Accession No. AF062402). rVb con-

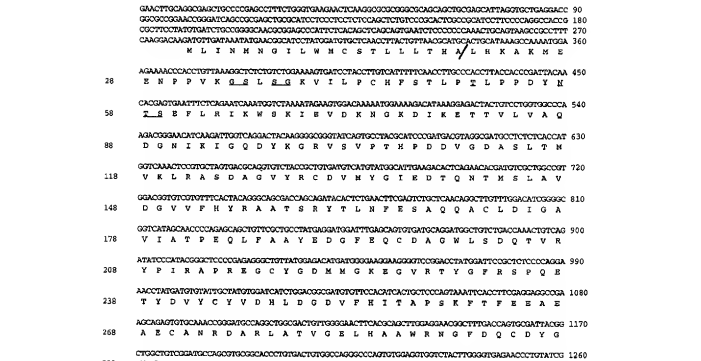

Figure 2c.

tains part of the αGAG domain, the βGAG domain, and the carboxy-terminal globular domain.

Structure of the V3 RNA

The Kozak[29] rule predicts the initiation of translation of rat versican at nucleotide 281, which is the position equivalent to that of mouse and human versican.[11,20] The 3′-untranslated regions (3′-UTR) of the 3 rat versican V3 cDNAs differed in length (147, 176, and 220 bases) (Figure 2C). This variation may reflect the actual lengths of the template molecules, or merely the binding of the oligo(dT) primer used for library synthesis to A-rich regions present in the 3′-UTR, rather than to the poly(A) tail. A polyadenylation consensus sequence is present only in the clone with the longest 3′-UTR (rVa) at position 2435 (Figure 2C).

Predicted Translation Product of V3

The predicted translation product of the V3 sequence is a 74474 Da polypeptide. Cleavage of a signal sequence is predicted after residue 20, and would result in a protein core of 72217 Da. As for the mouse,[31] an additional cysteine residue is present in the HABR, in comparison with human or chicken versican. O-link polysaccharide addition is predicted at threonine-51. The translated sequence includes 5 potential sites (NXS or NXT) for N-linked polysaccharide addition. CS chain addition could potentially occur at the SG or GS sequences indicated in Figure 2C; however, none of these sites match the D/EXSG consensus proposed by Bourdon.[31] Thus, V3 is likely to be a small glycoprotein and not a large proteoglycan.

Expression of V0 and V1 Versican Isoforms in Rat SMCs

All known versican isoforms contain the amino-terminal HABR and the G3 domain at the carboxy-terminus. Polyadenylated RNA was probed with PCR products amplified from those regions (Figure 3A). RNAs of 11 to 13 kb (smear), 9, 8.3, 7.7, 3.3, and 2.5 kb hybridized with both probes (Figure 3B, lanes 1 and 4). Thus, even the small RNAs contained amino- and carboxy-terminal sequences, implying that they are not degradation products of the larger bands. The 11- to 13-kb smear also hybridized to the αGAG and βGAG probes [lanes 2 and 3′ (a long exposure of lane 3)], identifying those molecules as V0 isoform. This variant has not been previously described in vascular SMCs. This finding was confirmed by cloning a partial V0 cDNA (see above). The 9-, 8.3-, and 7.7-kb RNAs hybridized to the βGAG (lane 3), but not the αGAG probes (lane 2), and thus are V1 isoform RNAs, in agreement with our previous findings in human and monkey SMCs.[2] No RNA hybridized to αGAG but not βGAG probes, indicating that SMCs do not express the V2 isoform. Each isoform is present as multiple RNA sizes. It is possible that these molecules are differentially spliced at other sites within the molecules. Mouse versican, however, has been shown to have multiple transcription stop signals, and the 3 large versican RNA bands (8, 9, and 10 kb), have been shown to vary in the length of the 3′-UTR.[9]

Expression of V3 in Rat SMCs

The 2 smallest versican RNA bands (3.3 and 2.5 kb) hybridized with both amino- and carboxy-terminal probes, but not the αGAG and βGAG probes (Figure 3B, lanes 1 through 4). Both bands also hybridized with an oligonucleotide (18VSPLR, see the Table) designed to be antisense to the junction between the amino- and carboxy-terminal versican globular domains (Figure 3B, lane 5). Because this junction is only present in the V3 isoform, both the 3.3- and 2.5-kb RNAs are V3 isoform RNA.

We further examined the expression of V3 in rat SMCs by RT-PCR analysis, using primers for the HABR and lectin-like domains. A band of the appropriate size (603 bp) was amplified from the V3 isoform plasmids, and from reverse-transcribed RNA from 2 lines of adult rat SMCs, but not from a rat pup SMC line (Figure 4B, left panel). This is in agreement with our previous finding that rat pup SMC lines express little or no versican RNA (of any size).[14] Bands corresponding to the larger, more abundant, versican isoforms were not detected, possibly because of the difficulty in either reverse-transcribing or amplifying across the large GAG-attachment exons. When a Southern blot of these reactions was probed with the splice junction oligonucleotide 18VSPLR, the oligonucleotide hybridized to the 603-bp bands (Figure 4B, right panel), confirming that they are derived from the V3 isoform.

Versican V3 Is Expressed by Human SMCs

Although we have previously examined the expression of versican RNA in human and monkey SMC total RNA, we have never detected the smaller bands (<7 kb) seen in the rat. To determine whether these forms were also present in human SMC RNA, we performed RT-PCR analysis to amplify from the HABR to either the 3′-UTR or the lectin-like domain (Figure 4A). Figure 4C shows that bands of the predicted sizes for the V3 isoform (750 and 1177 bp) can be reverse-transcribed from human SMC RNA (left panel, lanes 1 and 2). A PCR product of the appropriate size for V1, 383 bp, was produced by using the same forward primer and a reverse primer for the βGAG exon (Figure 4C, left panel, lane 3). The HABR/EGF splice junction oligonucleotide 19VS-PLH (see the Table) hybridized to the 1177- and 750-bp bands in lanes 1 and 2, but not to the 383-bp band (corresponding to V1) in lane 3, confirming that the first 2 bands represent the human V3 isoform (Figure 4C, right panel).

Cloning of Splice Variant Encoding a Different Carboxy-Terminus

To our knowledge, no differential splicing has been described within the region encoding the carboxy-terminal globular domain of versican. In a previous article, we uncovered preliminary evidence for the existence of an uncharacterized versican splice variant.[14] By RT-PCR analysis using primers within the carboxy-terminal region, we amplified 2 bands, where only 1 was predicted. The primers used were within the lectin-like domain and within the region after the CRP domain (Figure 5A). This latter region encodes the final 42 amino acids, and, together with the 3′-UTR, is encoded by the final exon in both mouse and humans; we will refer to this region as the "tail." In addition to the predicted 575-bp band, we amplified a band of ≈725 bp, suggesting the incorporation of additional unknown exon sequences into versican (Figure 5B, lanes 1 and 2). Bands of the same sizes were also amplified from human SMC RNA (Figure 5B, lanes 3 and 4).

To determine whether these PCR products represented a novel versican isoform, we cloned and sequenced the 725-bp bands (Figure 6A and 6B; Genbank Accession Nos. AF084544 and AF084545). The clones, rVint and hVint, contained sequences identical to rat and human versican, respectively. They also contained insertions of 145 bp (rat) and 144 bp (human) between the CRP and tail sequences, indicating the existence of a new splice variant (Figure 6B).

We compared the structure of the new clones with the published intron/exon structures of mouse and human versican. The CRP is encoded on a single exon, 14, and the tail is part of exon 15. Intron 14, between these exons, is very small in both species, and in humans its size is specified as 144 bp.[9,10] Thus, the new splice is an "unspliced" variant, where the last intron is not removed. In both the rat and human clones, consensus splice acceptor and splice donor elements

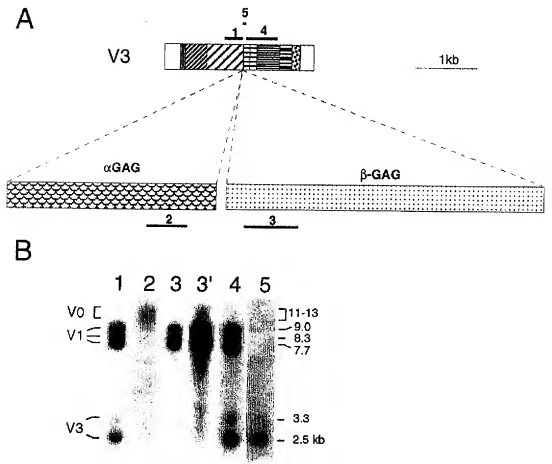

Figure 3. Characterization of rat versican RNAs. A, Diagram indicating the positions of the domain-specific probes on the rat versican splice variants. 1 indicates PCR product of rVd plasmid with oligonucleotides 16B' and 17B'; 2, αGAG sequences, a 632-bp *EcoRI/SacI* fragment of rVb; 3, βGAG sequences, an 834-bp *PstI/EcoRI* fragment of rVb; 4, PCR product of rVd with 21VE5 and 24VL3; and 5, an oligonucleotide (18VSPLR), antisense to the putative HABR/EGF splice junction in V3. B, Northern blot of poly(A)$^+$ RNA derived from adult rat SMC RNA line WKY3M-22. A single sample was run in 2 lanes on the same gel and the individual lanes were probed with the indicated probes. Lane numbers correspond to the probes diagrammed in A. 3' is a darker exposure of lane 3.

were present (see Figure 6A). The relative abundance of the 725-bp band and the 575-bp band in the original RT-PCR reaction suggested that we had not simply cloned a molecule in which splicing was incomplete, as we might expect such molecules to be relatively rare.

The predicted translation products of versican isoforms that retain intron 14 terminate within the intron (Figure 6C). Both human and rat translation products terminated after 16 amino acid residues, and showed a high sequence identity (81%). This may be compared with the 88% sequence identity between the rat and human tail regions. The net result of including intron sequences within the coding region of the carboxy-terminal globular domain would be to use this 16-amino acid region as the terminus, instead of the 42-amino acid tail, predicted from all previous versican cDNAs.

To determine whether the unspliced intron is expressed in versican RNAs containing or lacking the GAG attachment exons, RT-PCR was performed (see Figure 7A). PCR products of the appropriate size for V3 isoforms were amplified between the HABR primer and reverse primers within intron 14, or spanning the junction of exons 14 and 15, showing that V3 forms may retain or splice out intron 14 (Figure 7B, top). No bands representing V0 or V1 isoforms were detected in those reactions. This was expected, as PCR polymerization times (1 minute) were not long enough to amplify large molecules. By using a primer within the βGAG exon with either reverse primer, we determined that V1 isoform can also retain or splice out intron 14 (Figure 7B, bottom).

Discussion

We have shown that splice variants of versican are expressed by rat vascular SMCs in vitro. In our previous study, we showed that the V1 isoform is expressed in arteries in vivo and in vitro.[2,32] That study was completed before the discovery of versican splice variants and would not have detected the V3 isoform. In this study, we demonstrated that the V3 and V0 RNAs are also expressed by SMCs in vitro. We isolated 3 V3 cDNAs from an adult rat SMC cDNA library. We confirmed the existence of this splice variant in rat and human SMC RNA by RT-PCR, followed by hybridization to an oligonucleotide specific for this product. We also used this probe to detect V3 RNAs (3.3 and 2.5 kb) on northern blots.

In this study, we have asked only whether the V3 isoform is expressed by SMCs in vitro. Although it is possible that V3 is only expressed by cultured SMCs, which are known to dedifferentiated in culture, versican V3 RNA has been de-

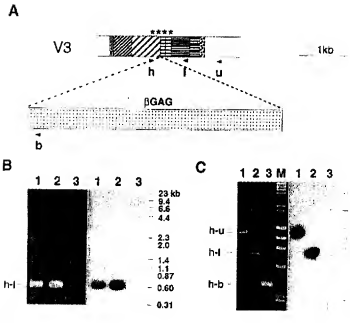

Figure 4. Expression of V3 isoform in adult rat SMCs and in human SMCs. A, Diagram showing position of PCR primers (h, b, l, and u) on versican (V1) and (V3) isoforms. ****Indicates the regions to which the antisense oligonucleotide could hybridize. B, Total RNA from rat SMC cultures was reverse-transcribed and was amplified by PCR. Lane 1, SD3 M-23 adult SMC line; lane 2, WKY3M-23 adult SMC line; and lane 3, WKY12-22 pup SMC line. The PCR primers (h, 09V; and l, 11V) were designed to amplify the region between the HABR and the lectin-like domain of versican. Left, Ethidium bromide–stained products of the RT-PCR reaction. Right, Southern blot of gel, probed with an oligonucleotide (18VSPLR), antisense to the putative HABR/EGF splice junction in V3. C, PCR of reverse-transcribed RNA from human SMCs. Human SMC RNA was reverse-transcribed and amplified by PCR from the HABR (primer h, 16B') to the following regions: lane 1, the 3'-UTR (primer u, 22VU3); lane 2, the lectin-like domain (primer l, 24VL3); lane 3, the βGAG region (primer b, 26VBG); lane M, size markers. Left, Ethidium bromide–stained PCR products. Right, Southern blot of the gel, probed with an oligonucleotide (19VSPLH), antisense to the putative HABR/EGF splice junction in V3.

tected in RNA isolated from several other tissues in vivo. V3 cDNA was cloned originally by PCR from mouse END-D cell cDNA library and a human cerebral cortex cDNA library.[12] The V3 mRNA from these sources was shown to be ≈3 kb by northern analysis.[12] V3 RNAs have also been detected by PCR from a mouse brain cDNA library, and human cerebral cortex, stomach, and fetal liver cDNA libraries, and by RT-PCR of human brain and brain tumor RNA.[12,13] Of these, only the END-D cell RNA is derived from cultured cells, and thus, V3 is expression is not restricted to dedifferentiated cultures. Nevertheless, changes in the pattern of splicing of other molecules have been shown to occur when smooth muscle cells undergo phenotypic modulation in culture.[34,35] It will be important to determine which versican splice variants are present in vivo and whether they change during disease processes.

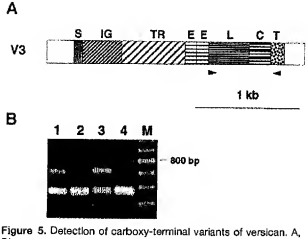

Figure 5. Detection of carboxy-terminal variants of versican. A, Diagram showing positions of the primers on the V3 isoform. B, RT-PCR of total RNA: lane 1, WKY3 M-23 adult rat SMC; and lane 3, human SMCs; and PCR of plasmid DNAs: lane 2, rat versican clone rVd; lane 4, human versican plasmid C10; and lane M, 100-bp ladder. Thirty cycles of amplification were performed, using primers in the lectin-like domain (27L) and the tail (10V).

The difference between the V0, V1, and V3 versican isoforms expressed by SMCs is in the presence or length of the central GAG attachment domains. The V0 core is longer than the V1 core (370 kDa vs 262 kDa for the human isoforms).[13,30] V1 has 12 to 15 putative GAG attachment sites whereas V0 has sites for an additional 5 to 8 chains.[13] The rat V3 cDNA sequence predicts a 72 kDa core lacking CS chains. Thus far, protein products have been demonstrated for the V0, V1, and V2 forms only, using antibodies specific for the αGAG and βGAG domains.[13,36,37] It is not known whether the V3 isoform has a protein product. Candidates for the V3 product are 2 small brain glycoproteins, glial hyaluronate-binding protein and hyaluronectin,[38,39] that were shown by protein sequence analysis to be products of the versican gene. These small hyaluronan-binding molecules appear to be either proteolytic products or small splice variants of versican.[38,40] Glial hyaluronate-binding protein appears to be a product of metalloproteinase digestion of the V1 isoform because its carboxy-terminus matches sequence within the βGAG exon and is identical to the terminus generated by metalloproteinase digestion of versican.[41] Both glial hyaluronate-binding protein and hyaluronectin are smaller than the predicted size of the V3 isoform. A protein product for the V3 isoform therefore has not yet been demonstrated. In our recent studies, however, we have overexpressed versican V3 RNA in rat SMCs and found alterations in cell shape and adhesion (Lemire et al, unpublished data, 1998). Thus, if V3 encodes a functional protein, it may have significant effects on cell phenotype.

The V0, V1, V2, and V3 isoforms have identical amino- and carboxy-termini. Functions shared by the 3 isoforms are likely to include hyaluronan binding via the amino-terminus, previously shown for the V1 isoform, and binding of tenascin-R, demonstrated for the lectin-like domain.[4,6,42] Other ligands may interact with the carboxy-terminal globular region via the EGF-like, CRP-like, or tail regions. Versican isoforms may therefore act as bridges of different lengths,[11–13] cross-linking matrix molecules.

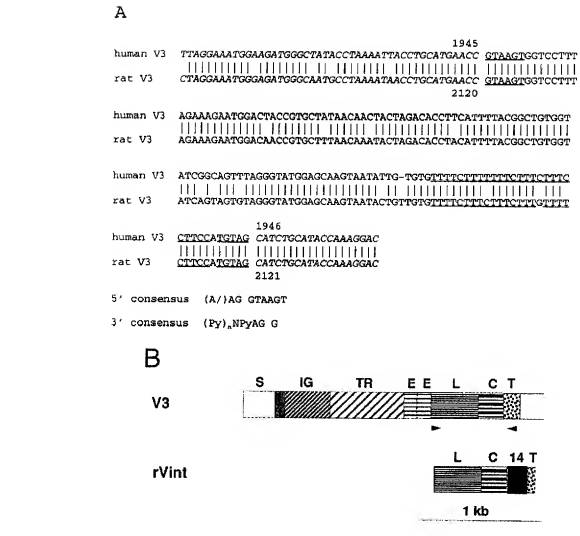

Figure 6. Sequence analysis of rat and human clones that are differentially spliced in the carboxy-terminal globular region. A, Sequence of the rat and human "intron" (in plain type) and adjacent sequences (italicized). Underlined sequences match the consensus for 5' and 3' splice elements indicated at the bottom. B, Diagram of the sequenced regions. C, Predicted translation products in the differentially spliced region. The CRP exon sequence is italicized.

The differences in the number of GAG attachment sites among the V0, V1, and V3 isoforms could also lead to significantly different roles in the vessel wall. The GAG chains are hygroscopic, and thus the V0 and V1 isoforms are large space-filling molecules that may contribute to vessel narrowing. The binding of LDL to versican's GAG chains may contribute to lipid deposition.[43] If the factors responsible for regulating the differential splicing could be understood, it may be possible to control the relative amounts of large and small versican isoforms, and thus prevent some of the pathological effects of excess versican synthesis. Alternatively, it may be possible to target the larger isoforms for destruction via the induction of specific proteolysis.

When considering possible functions for the versican isoforms, it may be important to consider not only their individual effects on the cells or tissues, but also the effect of 1 variant on the function of another. Our northern analysis suggests that the V3 RNA may be relatively abundant in smooth muscle; when poly(A)+ RNA was probed with 5' and 3' sequences present in all versican isoforms, the V3 RNA

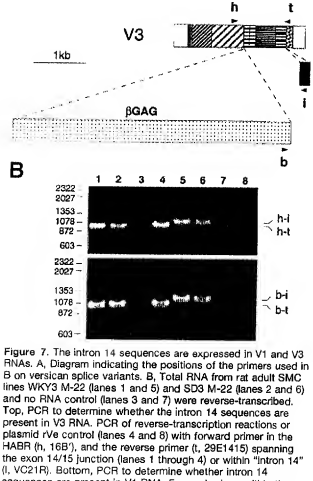

Figure 7. The intron 14 sequences are expressed in V1 and V3 RNAs. A, Diagram indicating the positions of the primers used in B on versican splice variants. B, Total RNA from rat adult SMC lines WKY3 M-22 (lanes 1 and 5) and SD3 M-22 (lanes 2 and 6) and no RNA control (lanes 3 and 7) were reverse-transcribed. Top, PCR to determine whether the intron 14 sequences are present in V3 RNA. PCR of reverse-transcription reactions or plasmid rVe control (lanes 4 and 8) with forward primer in the HABR (h, 16B') and the reverse primer (t, 29E1415) spanning the exon 14/15 junction (lanes 1 through 4) or within "intron 14" (I, VC21R). Bottom, PCR to determine whether intron 14 sequences are present in V1 RNA. Forward primer within the βGAG domain (b, 36BG) and reverse primers as for top. The plasmid control (lanes 4 and 8) for the bottom panel is rVb, a partial V0 isoform.

forms were only somewhat less abundant than the V1 forms. The identical amino-termini and carboxy-termini of the V0, V1, and V3 isoforms may compete for ligands. For example, do hyaluronate–versican aggregates containing both the small V3 isoforms (lacking CS chains) and the larger isoforms (having many CS chains) have a looser structure with less resistance to compression, when compared with aggregate formed with only larger variants? Are the carboxy-terminal selectin-like domains of the small variant inaccessible to ligands when that variant is part of an hyaluronate–aggregate containing V0 or V1?

We have also cloned a new variant that encodes a putative versican form having a different carboxy-terminus. We detected and cloned this variant by RT-PCR from both human and rat SMC RNA (note added in proof: while this article was in press, Perveen et al cloned the intron-containing versican variant from human fibroblasts. Perveen R, Hart-Holden N, Dixon MJ, Wiszniewski W, Fryer AE, Brunner HG, Pinkners AJLH, van Beersum SEC, Black GCM. Refined genetic and physical localization of the Wagner Disease (WGN1) locus and the genes CRTL1 and CSPG2 to a 2- to 2.5-cM region of chromosome 5q14.3. *Genomics.* 1999;57:219–226). This variant appears to be the product of incomplete splicing, and indeed it is possible that it represents a premature, nuclear RNA form. The relative abundance of this molecule, in comparison with the completely spliced form in RT-PCR experiments [Figures 5 and 7 and in RT-PCR using fewer amplification cycles (data not shown)], suggests it may be a functional mRNA. Similar "unspliced" or "optional" introns have been described in a few cases. The final introns are optional for the c-H-ras protooncogene, growth hormone, and 1 of the somatostatin receptors.[44–46] Retention of such introns appears to require poor matches with the splice site consensus sequences.[47] In the case of the versican intron 14, the consensus sequences within the intron match fairly well, but the adjacent sequences in exon 14 and 15 match poorly. Versican exon 15 also contains purine-rich sequences similar to those that function as splicing enhancers for optional introns.[48]

We have shown that versican intron 14 can be retained in both V1 and V3 variants. This unspliced form encodes a putative product lacking the 42 terminal residues of the original form and replacing those amino acids with a different 16-residue terminus. In both cases, the EGF-like, lectin-like, and CRP-like domains are retained. This is in agreement with previous studies that failed to find evidence for differential splicing of versican's G3 domains, but unlike the related molecule, aggrecan, which has splice forms lacking the EGF and CRP domains.[49]

Finally, what could be the role of the incompletely spliced isoform? It is possible that the carboxy-terminal tail may modify the binding properties of the adjacent CRP-like domain. The termini may alternatively have function independent of the proximate domain. The terminus of the form that splices out intron 14 (tail) has some (42% to 47%) homology to a family of intracellular protein tyrosine phosphatases.[28] The region of homology does not correspond to the active site and is proximal to the GLGF repeats. In a similar manner, the terminus of the form that retains intron 14 has only low homology to any known protein, in this case to snake venoms.[28] This homology is to the cysteine-rich region, which is hypothesized to modulate the activity of the disintegrin domain. Determining the functions of either of the 2 putative carboxy-termini will require further studies using antibodies and/or recombinant proteins.

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References

1. Wight TN. The vascular extracellular matrix. In: Fuster V, Ross R, Topol EJ, eds. *Atherosclerosis and Coronary Artery Disease.* New York, NY: Raven Press; 1996:421–440.
2. Yao LY, Moody C, Schonherr E, Wight TN, Sandell LJ. Identification of the proteoglycan versican in aorta and smooth muscle cells by DNA sequence analysis, in situ hybridization and immunohistochemistry. *Matrix Biol.* 1994;14:213–225.
3. Margolis RU, Margolis RK. Aggrecan-versican-neurocan family of proteoglycans. *Methods Enzymol.* 1994;245:105–126.
4. Aspberg A, Binkert C, Ruoslahti E. The versican C-type lectin domain recognizes the adhesion protein tenascin-R. *Proc Natl Acad Sci U S A.* 1995;92:10590–10594.
5. Ujita M, Shiromura T, Ito K, Kitagawa Y, Kimata K. Expression and binding activity of the carboxy-terminal portion of the core protein of

PG-M, a large chondroitin sulfate proteoglycan. *J Biol Chem.* 1994;269: 27603–27609.
6. Aspberg A, Miura R, Bourdoulous S, Shimonaka M, Heinegard D, Schachner M, Ruoslahti E, Yamaguchi Y. The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, binds tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc Natl Acad Sci U S A.* 1997;94:10116–10121.
7. Yamagata M, Saga S, Kato M, Bernfield M, Kimata K. Selective distributions of proteoglycans and their ligands in pericellular matrix of cultured fibroblasts: implications for their roles in cell-substratum adhesion. *J Cell Sci.* 1993;106:55–65.
8. Yamagata M, Suzuki S, Akiyama SK, Yamada KM, Kimata K. Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. *J Biol Chem.* 1989;264:8012–8018.
9. Shinomura T, Zako M, Ito K, Ujita M, Kimata K. The gene structure and organization of mouse PG-M, a large chondroitin sulfate proteoglycan: genomic background for the generation of multiple PG-M transcripts. *J Biol Chem.* 1995;270:10328–10333.
10. Naso MF, Zimmermann DR, Iozzo RV. Characterization of the complete genomic structure of the human versican gene and functional analysis of its promoter. *J Biol Chem.* 1994;269:32999–33008.
11. Ito K, Shinomura T, Zako M, Ujita M, Kimata K. Multiple forms of mouse PG-M, a large chondroitin sulfate proteoglycan generated by alternative splicing. *J Biol Chem.* 1995;270:958–965.
12. Zako M, Shinomura T, Ujita M, Ito K, Kimata K. Expression of PG-M (V3), an alternatively spliced form of PG-M without a chondroitin sulfate attachment region in mouse and human tissues. *J Biol Chem.* 1995;270: 3914–3918.
13. Doors-Zimmermann MT, Zimmermann DR. A novel glycosaminoglycan attachment domain identified in two alternative splice variants of human versican. *J Biol Chem.* 1994;269:32992–32998.
14. Lemire JM, Potter-Perigo S, Hall KL, Wight TN, Schwartz SM. Distinct rat aortic smooth muscle cells differ in versican (PG-M) expression. *Arterioscler Thromb Vasc Biol.* 1996;16:821–829.
15. Lemire JM, Covin CW, White S, Giachelli CM, Schwartz SM. Characterization of cloned aortic smooth muscle cells from young rats. *Am J Pathol.* 1994;144:1068–1081.
16. Giachelli CM, Majesky MW, Schwartz SM. Developmentally regulated cytochrome P-450IA1 expression in cultured rat vascular smooth muscle cells. *J Biol Chem.* 1991;266:3981–3986.
17. Gordon D, Mohai LG, Schwartz SM. Induction of polyploidy in cultures of neonatal rat aortic smooth muscle cells. *Circ Res.* 1986;59:633–644.
18. Majesky MW, Bendtt EP, Schwartz SM. Expression and developmental control of platelet-derived growth factor A-chain and B-chain/Sis genes in rat aortic smooth muscle cells. *Proc Natl Acad Sci U S A.* 1988;85:1524–1528.
19. Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell.* 1990;63:515–524.
20. Chomczynski P, Sacchi N. Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987;162:156–159.
21. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
22. Schonherr E, Kinsella MG, Wight TN. Genistein selectively inhibits platelet-derived growth factor stimulated versican biosynthesis in monkey arterial smooth muscle cells. *Archiv Biochem Biophys.* 1997;339: 353–361.
23. Genetics Computer Group (GCG). Wisconsin Package. 9.1 ed. Madison, WI: Genetics Computer Group (GCG); 1997.
24. Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 1997;10:1–6.
25. Hansen JE, Lund O, Engelbrecht J, Bohr H, Nielsen JO, Hansen J-ES, Brunak S. Prediction of O-glycosylation of mammalian proteins: specificity patterns of UDP-GalNAc:-polypeptide *N*-acetylgalactosaminyltransferase. *Biochem J.* 1995;308:801–813.
26. Hansen JE, Lund O, Rapacki K, Brunak S. O-GLYCBASE version 2.0: a revised database of O-glycosylated proteins. *Nucleic Acids Res.* 1997; 25:278–282.
27. Hansen JE, Lund O, Tolstrup N, Gooley AA, Williams KL, Brunak S. NetOglyc: prediction of mucin type O-glycosylation sites based on sequence context and surface accessibility. *Glycoconjugate J.* 1998;15: 115–130.
28. Wortey KC, Wiese BA, Smith RF. BEAUTY: an enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity results. *Genome Res.* 1995;5:173–184.
29. Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 1987;15:8125–8148.
30. Zimmermann DR, Ruoslahti E. Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J.* 1989;8:2975–2981.
31. Bourdon MA. Structure and role of cloned small proteoglycans. In: Sandell L, Boyd C, eds. *Extracellular Matrix Genes.* San Diego, CA: Academic Press; 1990:157–174.
32. Schonherr E, Jarvelainen HT, Sandell LJ, Wight TN. Effects of platelet-derived growth factor and transforming growth factor-beta 1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J Biol Chem.* 1991;266:17640–17647.
33. Paulus W, Baur I, Doors-Zimmermann MT, Zimmermann DR. Differential expression of versican isoforms in brain tumors. *J Neuropathol Exp Neurol.* 1996;55:528–533.
34. Kashiwada K, Nishida W, Hayashi K, Ozawa K, Yamanaka Y, Saga H, Yamashida T, Tohyama M, Shimada S, Sato K, Sobue K. Coordinate expression of alpha-tropomyosin and calsdemon isoforms in association with phenotypic modulation of smooth muscle cells. *J Biol Chem.* 1997; 272:15396–15404.
35. Glukhova MA, Frid MG, Shchonin BV, Vasilevskaya TD, Grunwald J, Saginati M, Kotelianskiy VE. Expression of extra domain A fibronectin sequence in vascular smooth muscle cells is phenotype dependent. *J Cell Biol.* 1989;109:357–366.
36. Shinomura T, Nishida Y, Ito K, Kimata K. cDNA cloning of PG-M, a large chondroitin sulfate proteoglycan expressed during chondrogenesis in chick limb buds: alternative spliced multiforms of PG-M and their relationships to versican. *J Biol Chem.* 1993;268:14461–14469.
37. Zako M, Shinomura T, Kimata K. Alternative splicing of the unique "plus" domain of chicken PG-M/versican is developmentally regulated. *J Biol Chem.* 1997;272:9325–9331.
38. Perides G, Lane WS, Andrews D, Dahl D, Bignami A. Isolation and partial characterization of a glial hyaluronate-binding protein. *J Biol Chem.* 1989;264:5981–5987.
39. Delpech B, Delpech A, Bruckner G, Girard N, Maingonnat C. Hyaluron and hyaluronectin in the nervous system. In: Evered D (organizer), Whelan J, eds. *The Biology of Hyaluronan.* CIBA Foundation Symposium Vol 143. Chichester, UK: John Wiley; 1989:208–232.
40. Delpech B, Girard N, Olivier A, Maingonnat C, van Driessche G, van Beeumen J, Bertrand P, Duval C, Delpech A, Bourguignon J. The origin of hyaluronectin in human tumors. *Int J Cancer.* 1997;72:942–948.
41. Perides G, Asher RA, Lark MW, Lane WS, Robinson RA, Bignami A. Glial hyaluronate-binding protein: a product of metalloproteinase digestion of versican? *Biochem J.* 1995;312:377–384.
42. LeBaron RG, Zimmermann DR, Ruoslahti E. Hyaluronate binding properties of versican. *J Biol Chem.* 1992;267:10003–10010.
43. Camejo G, Hurt Camejo E, Olsson U, Bondjers G. Proteoglycans and lipoproteins in atherosclerosis. *Curr Opin Lipidol.* 1993;4:385–391.
44. Hwang D-Y, Cohen JB. A splicing enhancer in the 3'-terminal c-H-*ras* exon influences mRNA abundance and transforming activity. *J Virol.* 1997,71:6416–6426.
45. Hampson RK, Rottman FM. Alternative processing of bovine growth hormone mRNA: nonsplicing of the final intron predicts a high molecular weight variant of bovine growth hormone. *Proc Natl Acad Sci U S A.* 1987;84:2673–2677.
46. Vanetti M, Kouba M, Wang X, Vogt G, Holtt V. Cloning and expression of a novel mouse somatostatin receptor. *FEBS Lett.* 1992;311:290–294.
47. Watakabe A, Tanaka K, Shinomura Y. The role of exon sequences in splice site selection. *Genes Dev.* 1993;7:407–418.
48. Dirksen WP, Sun Q, Rottman FM. Multiple splicing signals control alternative intron retention of bovine growth hormone pre-mRNA. *J Biol Chem.* 1995;270:5346–5352.
49. Grover J, Roughley PJ. Versican gene expression in human articular cartilage and comparison of mRNA splicing variation with aggrecan. *Biochem J.* 1993;291:361–367.